REGULATION OF PYRUVATE METABOLISM BY THE MITOCHONDRIAL ENERGY STATE: THE EFFECT OF PALMITYL-COENZYME A

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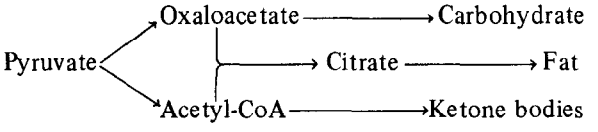
1. Introduction

In isolated rat liver mitochondria pyruvate can either be decarboxylated or carboxylated. In the former case the pyruvate dehydrogenase complex (EC 1.2.4.1) oxidizes pyruvate to acetyl-CoA (AcCoA) leading to citrate synthesis or ketone-body formation. In the latter case pyruvate is converted to oxaloacetate by the action of pyruvate carboxylase (EC 6.4.1.1). Oxaloacetate may enter the gluconeogenic pathway by its conversion into phosphoenolpyruvate. Alternatively, it may depress ketogenesis by diverting AcCoA towards the synthesis of citrate which is considered to be a lipogenic precursor [1].



From the scheme it is evident that the control of mitochondrial pyruvate metabolism is of key importance in the regulation of gluconeogenesis, ketogenesis and lipogenesis in liver [2, 3].

Several factors have been reported to modify the activities of isolated pyruvate dehydrogenase [4–7] and pyruvate carboxylase [8–11]. Increasing AcCoA/CoA, ATP/ADP and NADH/NAD^+ ratios may cooperatively switch mitochondrial pyruvate metabolism from decarboxylation to carboxylation [12]. This regulatory pattern has been confirmed in isolated liver mitochondria [13–17].

Recently the interesting suggestion was brought forward that pyruvate metabolism is affected indirectly by the level of long-chain acyl-CoA esters. Long-chain acyl-CoA esters like palmityl-CoA strongly inhibit adenine nucleotide (AdN) translocation through the inner mitochondrial membrane [18–23]. A reduced rate of AdN transport would enhance the intramitochondrial ATP/ADP ratio since the maximal rates of AdN transport and ATP synthesis are of the same order of magnitude [24]. As mentioned above, the ATP/ADP ratio is one of the factors controlling the activity of pyruvate carboxylase [16, 17]. Consequently, the level of acyl-CoA esters may be of importance in the control of pyruvate carboxylation *in vivo*. This effect of acyl-CoA esters would be comparable to their stimulatory effect on ketogenesis in liver mitochondria described earlier [25].

In the present report the effect of State-3 to State-4 transitions on pyruvate metabolism is compared with the effect of palmityl-CoA. Our results are compatible with the conclusion of Walter and coworkers [16, 17] that, at optimal substrate concentrations, pyruvate carboxylation is mainly regulated by the phosphorylation state of the AdN. At elevated ATP/ADP ratios pyruvate oxidation is diminished whereas pyruvate carboxylation is stimulated leading to an accumulation of Krebs-cycle intermediates and a lowered ketone-body formation from pyruvate. A similar stimulation of pyruvate carboxylation can be brought about by palmityl-CoA, presumably due to an inhibition of AdN translocation and a concomitant increase in the mitochondrial ATP/ADP ratio.

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2. Methods and materials

Oxygen uptake was measured at 25° with a Gilson Respirometer. The standard reaction medium contained 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 50 mM Tris-HCl, 30 mM potassium phosphate, 30 mM glucose, 0.5 mM ADP and 25 mM $KHCO_3$, pH 7.5. Reactions were started by the addition of the mitochondria. Final volume, 2.0 ml. After 16 min 1 ml ice-cold $HClO_4$ (1.2 N) was added and metabolites were assayed spectrophotometrically in the deproteinized supernatants using standard enzymic methods.

Pyruvate carboxylation was measured as the increase in malate, fumarate and citrate. It has been shown [26] that over 90% of the pyruvate carboxylated is recovered in these intermediates. Addition of fluorocitrate to block the oxidation of citrate proved to be less satisfactory. In our hands 200 μM potassium fluorocitrate was needed to inhibit completely the oxidation of added citrate. At this concentration of fluorocitrate, however, a 50% inhibition of pyruvate carboxylation was observed.

The relative rate of ketogenesis is expressed as the *acetyl-ratio* [27], i.e. the quotient of AcCoA converted into ketone bodies and the total flux through the AcCoA pool:

$$\text{acetyl-ratio} = \frac{2 \times \text{ketone bodies formed}}{\text{pyruvate used} - \text{pyruvate carboxylated}}$$

The 3-hydroxybutyrate/acetoacetate (HB/Acac) ratio is used as an indicator of the mitochondrial NADH/NAD^+ ratio [28].

L-Palmitylcarnitine was synthesized according to Bremer [29]. L-Carnitine chloride was a generous gift of Otsuka Pharm. Fact. (Osaka). Palmityl-CoA was obtained from Serva; barium fluorocitrate and carbonyl-cyanide-*m*-chlorophenylhydrazone (CCCP) from Calbiochem. Other biochemicals and experimental details were exactly as previously described [20, 25].

3. Results and discussion

3.1. Influence of the mitochondrial energy state on pyruvate metabolism

As can be seen from table 1, under State-4 conditions (hexokinase absent) in the presence of 25 mM $KHCO_3$ about half of the pyruvate metabolized is carboxylated and less than 10% is converted into ketone bodies. Under State-3 conditions (hexokinase added) with added bicarbonate pyruvate carboxylation is markedly depressed and parallel increases in pyruvate oxidation and in ketone-body formation are observed. Virtually no pyruvate carboxylation occurs in a State-3 medium without $KHCO_3$. It is noteworthy that added malate also inhibits pyruvate carboxylation. This inhibition may be explained either by a direct effect of malate (or oxaloacetate) on pyruvate carboxylase or by an efficient removal of AcCoA which is an activator of pyruvate carboxylase [8–11].

Table 1
Effects of hexokinase and L-malate on pyruvate metabolism.

Additions	Pyruvate: Used	Carboxylated	Converted into ketone bodies	Carboxylated / Decarboxylated	HB / Acac
	(μmoles)				
None	4.84	2.37	0.44	0.96	0.47
Hexokinase	3.67	0.54	1.18	0.17	0.16
None†	2.15	0.40	1.22	0.23	0.65
Hexokinase†	2.62	0.08	1.40	0.03	0.13
L-Malate	4.22	1.58	0.26	0.60	0.44

† In these experiments $KHCO_3$ was replaced by an equimolar concentration of KCl.

The standard reaction medium (see Methods) was supplemented with 3 mM pyruvate. Further additions: 2.6 units of hexokinase, 1 mM L-malate. Mitochondrial protein, 11.0 mg.

In fig. 1 the time courses of pyruvate carboxylation and of ketogenesis are compared in various energy states. Obviously, the acetyl-ratio (see Methods) is inversely related to the rate of pyruvate carboxylation. In uncoupled mitochondria the accumulation of Krebs-cycle intermediates is negligible and the acetyl-ratio increases to about 0.75. On the other hand, under State-4 conditions pyruvate carboxylation is an active process and the acetyl-ratio decreases from 0.30 to about 0.15.

The findings of table 1 and fig. 1 support the conclusion of Walter and coworkers [16, 17] that under these *in vitro* conditions the rate of pyruvate carboxylation is mainly determined by the phosphorylation

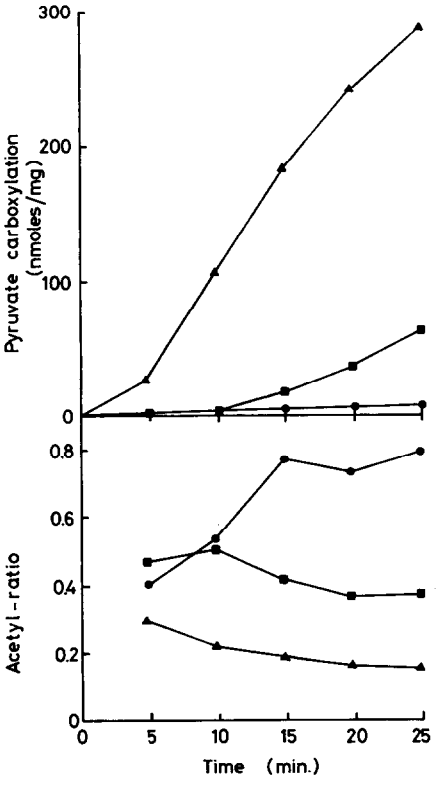

Fig. 1. Time course of pyruvate carboxylation and ketogenesis in various energy states. The standard reaction medium was supplemented with 5 mM pyruvate. Further additions as indicated below. After addition of the mitochondria 15.0 ml incubations were shaken vigorously in open Erlenmeyer flasks at 25°. At the times indicated 2.0 ml aliquots were withdrawn and analyzed. Mitochondrial protein, 4–6 mg/ml reaction medium. (▲—▲—▲) No further additions (State-4); (■—■—■) + hexokinase, 1.3 units/ml (State-3); (●—●—●) + hexokinase (1.3 units/ml) + 1 μM CCCP.

state of the mitochondrial AdN. It should be noted however, that the *in vivo* regulation of pyruvate carboxylation is still a matter of debate [cf. 9,10].

3.2. Ketogenesis: Comparison of pyruvate and fatty acids as precursors

It follows from the preceding section that the concentration of Krebs-cycle intermediates is an important factor in the control of ketogenesis from pyruvate, whereas the energy state *per se* has only a minor influence. On the other hand, in the case of ketogenesis from fatty acids both the concentration of Krebs-cycle intermediates and the energy state are important parameters in the control of the acetyl-ratio [27, 30]. This discrepancy between fatty-acid and pyruvate oxidation may be explained by the observation that during pyruvate oxidation in State-4 the HB/Acac ratio is lower than unity (table 1), whereas during palmitylcarnitine oxidation this ratio may reach values of five or higher [27]. An increase of the mitochondrial $NADH/NAD^+$ ratio lowers the level of oxaloacetate, thereby limiting the activity of citrate synthase and stimulating ketogenesis [31; cf. 32].

The lower HB/Acac ratios observed during pyruvate oxidation as compared with palmitylcarnitine oxidation may be caused by several factors: i) A continuous consumption of ATP in the carboxylation of pyruvate; ii) A low capacity of pyruvate dehydrogenase to produce AcCoA and reducing equivalents as compared with the β-oxidation of fatty acids; and iii) Consumption of NADH in the formation of malate from oxaloacetate following pyruvate carboxylation.

In short, we propose that a State-3 to State-4 transition lowers ketogenesis from pyruvate by activating pyruvate carboxylase. The newly formed oxaloacetate diverts AcCoA from acetoacetate synthesis to the formation of citrate. On the other hand, in the case of fatty-acid oxidation ketogenesis is stimulated when going from a low to a high energy state. This stimulation reflects the decreased intramitochondrial oxaloacetate concentration caused by the drastically elevated $NADH/NAD^+$ ratio during fatty-acid oxidation in State-4.

3.3. Effects of fatty acid oxidation and palmityl-CoA on pyruvate metabolism

Upon addition of increasing amounts of palmityl-CoA to mitochondria oxidizing pyruvate under State-3 conditions, parallel increases in the ratio of pyruvate carboxylation over pyruvate decarboxylation and the HB/Acac ratio are observed (table 2). Either a suboptimal amount of atractylate or the omission of hexokinase have analogous effects. The inhibition of mitochondrial AdN transport by palmityl-CoA leads to an increase of the mitochondrial phosphate potential, which in turn enhances the rate of pyruvate carboxylation. This stimulation of pyruvate carboxylation by palmityl-CoA is still more striking in the presence of

Table 2
Effects of added palmityl-CoA on pyruvate metabolism.

Additions	Oxygen uptake (μmoles)	Pyruvate			HB / Acac
		Used (μmoles)	Carboxylated	Carboxylated / Decarboxylated	
None	3.7	3.00	0.22	0.08	0.05
PCoA (2 nmoles/mg)	3.6	3.39	0.34	0.11	0.13
PCoA (5 nmoles/mg)	4.2	3.74	0.60	0.19	0.17
PCoA (10 nmoles/mg)	3.2	4.07	1.31	0.47	0.37
Atractylate	4.1	3.67	0.59	0.19	0.15
None†	2.5	4.67	2.37	1.03	0.44
Palmitate	5.6	2.72	0.38	0.16	0.08
Palmitate + PCoA (5 nmoles/mg)	5.9	3.54	1.47	0.71	0.26

† Hexokinase was omitted in this experiment.
To the standard reaction medium 5.2 units of hexokinase and 3 mM pyruvate were added. Further additions: palmityl-CoA (PCoA) as indicated, 2.0 μg atractylate, 0.5 mM palmitate. Mitochondrial protein, 10.3 mg.

Table 3
Stimulation of pyruvate carboxylation by palmityl-CoA generated from palmitylcarnitine.

Additions	Pyruvate			HB / Acac
	Used (μmoles)	Carboxylated	Carboxylated / Decarboxylated	
None	3.89	0.21	0.06	0.10
Palmitate	3.46	0.32	0.10	0.07
CoASH (100 μM)	3.91	0.33	0.09	0.11
Palmitate + CoASH (100 μM)	3.65	1.17	0.47	0.51
Palmitate + CoASH (100 μM) + Cn	3.74	0.47	0.14	0.10
PCn	2.46	0.94	0.62	0.16
PCn + CoASH (20 μM)	2.62	1.34	1.05	0.36

The standard reaction medium was supplemented with 2.6 units of hexokinase and 3 mM pyruvate. Further additions: 0.1 mM palmitate, CoASH as indicated, 0.5 mM L-carnitine (Cn), 0.5 mM L-palmitylcarnitine (PCn). Mitochondrial protein, 11.3 mg.

an additional amount of palmitate. In view of the observed increase in oxygen uptake, this effect of added palmitate can be explained by the stimulation of oxidative phosphorylation as a consequence of palmitate oxidation. This will elevate the mitochondrial ATP/ADP ratio even further. A similar effect of oleyl-CoA was reported by Stucki et al. [17].

In the experiments shown in table 3, palmityl-CoA was generated during the incubations from either palmitate + CoASH or L-palmitylcarnitine + CoASH [25]. In the former case palmityl-CoA is formed by the palmityl-CoA synthetase localized in the outer mitochondrial membrane [33], in the latter case by carnitine palmityltransferase [34]. In both cases extra-

mitochondrial palmityl-CoA accumulates due to the impermeability of the inner mitochondrial membrane towards acyl-CoA and CoASH [35]. The results in table 3 are entirely consistent with the effects of added palmityl-CoA (table 2) and they also show that L-carnitine counteracts these effects. Parenthetically, it may be noted that palmitate (table 2) as well as palmitylcarnitine (table 3) lower the rate of pyruvate decarboxylation in agreement with the well-documented sparing effect of fatty acids on pyruvate oxidation.

Halperin et al. [36] recently reported that palmityl-CoA has an inhibitory effect on the translocation of citrate and malate in rat liver mitochondria. However, in our experiments no interference with the accumulation of malate, fumarate and citrate (the main products of pyruvate carboxylation) could be detected, showing that in our system the AdN translocation rather than the transport of these Krebs-cycle intermediates is rate-limiting.

In conclusion, the following effects occur when palmitate is added to mitochondria oxidizing pyruvate: i) An acceleration of ATP synthesis; and ii) Accumulation of palmityl-CoA slowing down the exchange of AdN through the inner mitochondrial membrane. Both effects will cause an increase in the intramitochondrial ATP/ADP ratio and a concomitant stimulation of pyruvate carboxylation. In addition, the mitochondrial pool of exchangeable nucleotides will be lowered by an increased AMP level due to intramitochondrial palmitate activation [17]. A regulatory role of the AcCoA/CoA ratio [cf. 8–11] remains to be established.

The present investigation points to a possible role of long-chain acyl-CoA esters in the regulation of gluconeogenesis in rat liver. Arguments in favour of this hypothesis [37, 25] may be briefly summarized as follows: i) The increased levels of long-chain acyl-CoA observed *in vivo* during starvation [37, 38] simultaneously with a stimulated gluconeogenesis and ketogenesis; ii) The competitive inhibition of the AdN translocator by low levels of long-chain acyl-CoA and its reversal by albumin and L-carnitine [18–23]; iii) The increase in the mitochondrial phosphate potential [37] and the decrease of the cytosolic one [39] calculated to occur in starvation and in starvation followed by refeeding fat; and iv) The stimulation by palmityl-CoA of ketogenesis during palmitate oxidation [25] and of pyruvate carboxylation (present results) in isolated rat-liver mitochondria.

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